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$p27^{Xic1}$, a Cdk Inhibitor, Promotes the Determination of Glial Cells in *Xenopus* Retina

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Summary

$p27^{Xic1}$, a member of the Cip/Kip family of Cdk inhibitors, besides its known function of inhibiting cell division, induces Müller glia from retinoblasts. This novel gliogenic function of $p27^{Xic1}$ is mediated by part of the N-terminal domain near but distinct from the region that inhibits cyclin-dependent kinases. Cotransfections with dominant-negative and constitutively active Delta and Notch constructs indicate that the gliogenic effects of $p27^{Xic1}$ work within the context of an active Notch pathway. The gradual increase of $p27^{Xic1}$ in the developing retina thus not only limits the number of retinal cells but also increasingly favors the fate of the last cell type to be born in the retina, the Müller glia.

Introduction

In the retina and the cortex, there is a well-defined histogenetic order of laminar fates, but investigations into cell cycle aspects of fate decision in these tissues have led to a variety of conclusions about when in the cell cycle fate is determined (Cepko, 1999; Edlund and Jessell, 1999). For example, the laminar fate of cortical progenitor cells must be acquired by S phase of final cell division (McConnell and Kaznowski, 1991; Bohner et al., 1997). Retinal progenitors lose their competence to respond to an amacrine inhibitory factor, and motor neuron precursors lose their competence to respond to Sonic Hedgehog, once they enter M phase in their final cell division (Ericson et al., 1996; Belliveau and Cepko, 1999). The mechanisms that relate the cell cycle to such determination events are unknown. One possibility is that certain phases of the cell cycle provide a passive environment in which determination factors work. Alternatively, it may be that molecules involved in cell cycle actively collaborate in cellular determination.

The cyclin-dependent kinase (Cdk) inhibitors may have particular significance in determination, as the patterns of expression of these genes are coordinated with histogenesis. Mice lacking both $p21^{Cip1}$ and $p57^{Kip2}$ display severe defects in formation of myotubes similar to that of mice lacking the myogenic factor, myogenin (Zhang et al., 1999). $p27^{Kip1}$ and $p57^{Kip2}$ are also involved in lens, placenta, and lung differentiation (Zhang et al., 1998, 1999). In the nervous system, $p27^{Kip1}$ is expressed in the whole mouse retina at E15.5, as cells differentiate (Zhang et al., 1998), and later in the oligodendrocyte precursors of the optic nerve during their differentiation. Interestingly, mice lacking $p27^{Kip1}$ have enlarged organs, including extra cells in the retina. $p27^{Kip1}$ null mice also show disrupted lamination of the retina and an increased number of oligodendrocytes (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996), while overexpression of $p27^{Kip1}$ in O2-A progenitors results in premature cell cycle arrest (Casaccia-Bonnefil et al., 1997; Tikoo et al., 1997, 1998; Durand et al., 1998; Raff et al., 1998).

Retinal neuroblasts are multipotent and can give rise to different combinations of cell types even late in retinogenesis (Price et al., 1987; Turner and Cepko, 1987; Holt et al., 1988; Wetts and Fraser, 1988; Turner et al., 1990; Jensen and Raff, 1997). This indeterminacy is coupled to a conserved histogenetic order. Retinal ganglion cells and horizontal cells are born first, followed by cone photoreceptor cells, amacrine cells, rod photoreceptor cells, and bipolar cells. Finally, Müller cells are born and differentiate (Cepko, 1999). Activation of the Notch pathway prevents cellular differentiation in the retina, while release of inhibition by the expression of a dominant-negative form of Delta, Delta-stu, causes an increase of early born cell types at the expense of later born cell types (Dorsky et al., 1997; Henrique et al., 1997). The effects of these genes have not been linked to the cell cycle, although their expression patterns suggest coordination of some sort. In *Xenopus*, many activators of the cell cycle have been identified, and their functions on early embryonic cell division have been studied. A Cdk inhibitor of the Cip/Kip family, *$p27^{Xic1}$* (Su et al., 1995), and its possible allele, *$p28^{Kix1}$* (Shou and Dunphy, 1996), have also been cloned. Here we report that $p27^{Xic1}$ overexpression can not only block proliferation in the retina but can also initiate the early differentiation of the last cell type to be born, the Müller cells.

Results

Expression of *$p27^{Xic1}$* Gradually Increases during Retinal Development

$p27^{Xic1}$ expression first becomes obvious during gastrula stages as diffuse staining throughout the epidermis. Expression becomes more intense as development progresses and is evident in the myotome at early tail bud stages (stages 22–23, Figure 1A). In the eye, $p27^{Xic1}$ is first detected at stage 26, when retinal ganglion cells start to differentiate (Holt et al., 1988) (Figure 1B). As *$p27^{Xic1}$* expression in the myotome decreases, the expression in the eye increases, and at late tail bud stage

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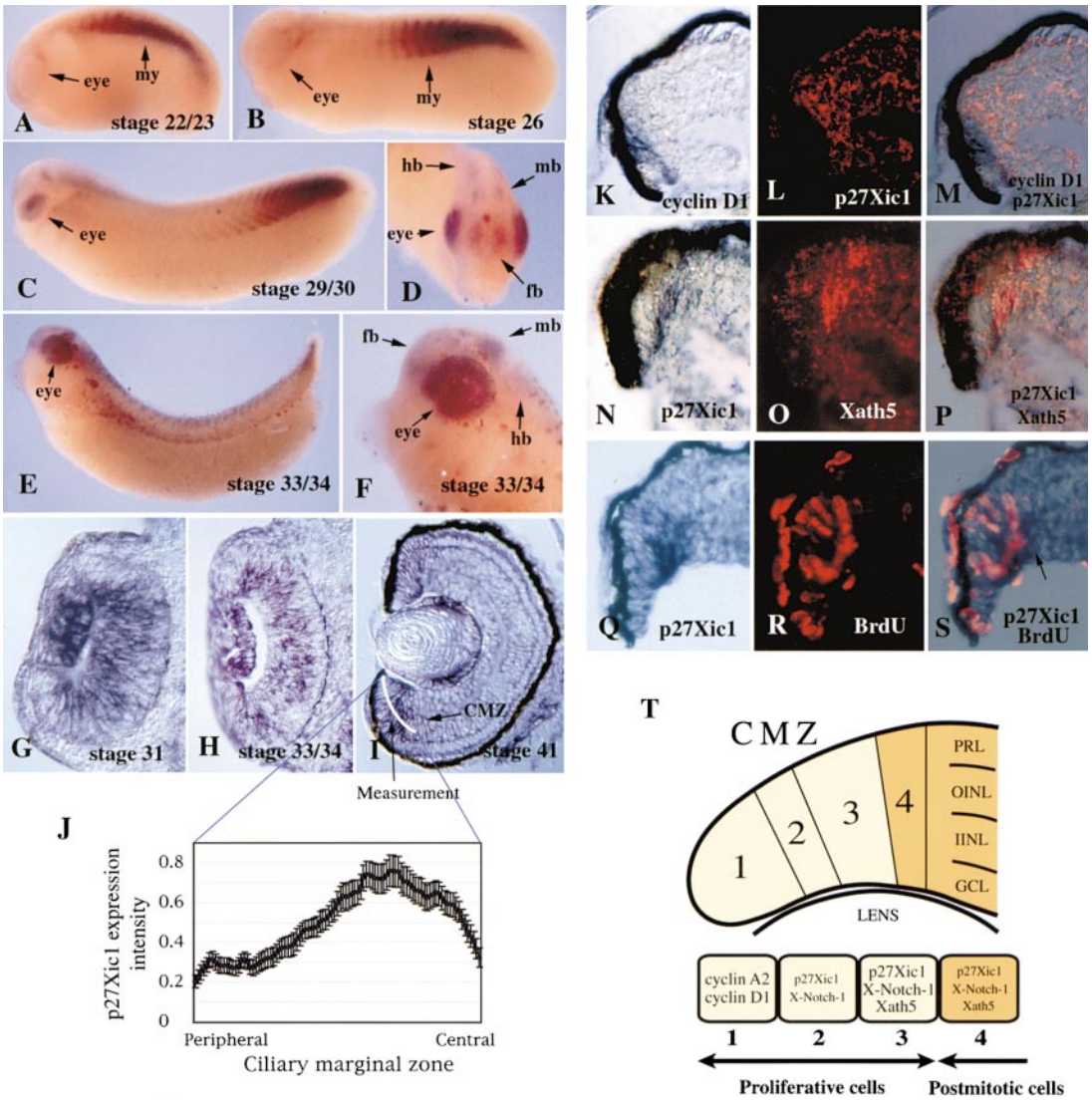

Figure 1. Temporal and Regional Expression Pattern of p27^{Xic1}

(A–F) Whole-mount in situ hybridizations with *p27^{Xic1}* at stage 22/23 (A), stage 26 (B), stage 29/31 (C and D), and stage 33/34 (E and F). Lateral views (A, B, C, E, and F). Anterior view (D). Enlarged view of (E) (F). my, myotome, hb, hindbrain, mb, midbrain, fb, forebrain.

(G) In a section of a stage 31 retina, ubiquitous expression of p27^{Xic1} was seen in columnar neuroepithelial cells at central retina.

(H) Later, at stage 33/34, the expressed region became gradually restricted in the middle layer of retina and the CMZ.

(I) Finally, at stage 41, the expression was mainly restricted in the CMZ with weak expression in the central retina.

(J) Measurement of p27^{Xic1} expression in CMZ. After the in situ hybridization with *p27^{Xic1}*, the density of staining in CMZs of 38 retinas was analyzed by NIH image software. The measured region is indicated in (I) as a white line. Averages and SEMs of 100 sampling points are shown.

(K–M) Double in situ hybridizations were performed with DIG-labeled *cyclin D1* (K) and fluorescein-labeled *p27^{Xic1}* (L). (M) Overlapped image of (K) and (L).

(N–P) Double in situ hybridizations were performed on sections of *Xenopus* retina at stage 41 using DIG-labeled *p27^{Xic1}* (N) and fluorescein-labeled *Xath5* (O). (P) Overlapped image of (N) and (O).

(Q–S) Double staining for BrdU uptake and *p27^{Xic1}* expression. (Q) In situ hybridization with *p27^{Xic1}*. (R) BrdU immunostaining. (S) Overlapped image of (Q) and (R) (arrow points to p27^{Xic1}-positive BrdU-negative central area).

(T) Schematic representation of *cyclin A1*, *cyclin D1*, *X-Notch-1*, *p27^{Xic1}*, and *Xath5* in the CMZ.

(stage 29/30), *p27^{Xic1}* is expressed throughout most of the retina (Figures 1C and 1D). p27^{Xic1} is also found in all three brain vesicles. At stage 32 to 34, *p27^{Xic1}* is highly expressed in the central retina (Figures 1E, 1F, and 1H). By stage 41, levels drop in the central retina and strong *p27^{Xic1}* expression is largely restricted to the ciliary marginal zone (CMZ), where retinogenesis continues throughout life (Figure 1I). Quantitative RT–PCR from developing eyes shows a 3-fold increase in p27^{Xic1} RNA from stage 25 to stage 35/36, followed by a reduction to the original level by stage 41 (data not shown).

In the CMZ, the spatial gradient from peripheral to central regions recapitulates the temporal progression of retinal development. Molecules expressed more peripherally in the CMZ are also expressed earlier in retinogenesis (Perron et al., 1998). Therefore, we measured

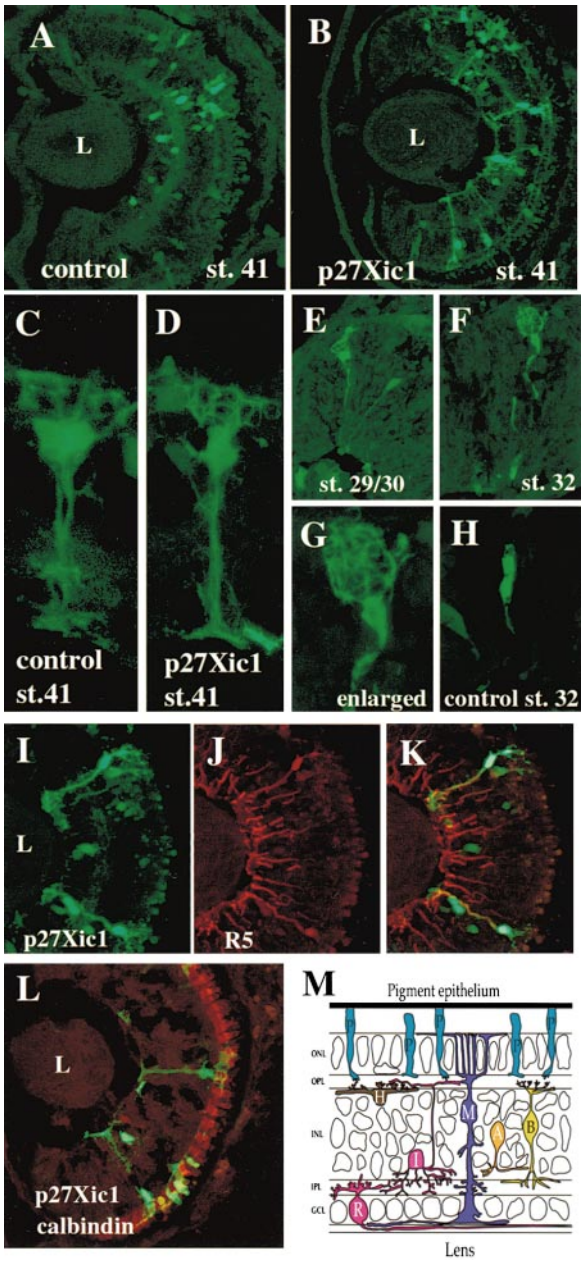

Figure 2. p27Xic1-Overexpressing Cells Differentiate into Müller Glia

Embryos were lipofected at stage 15 in the eye primordia with an expression construct of *p27Xic1* plus pGFP or pGFP alone (control). After fixation at the indicated stage, the embryos were cryostat sectioned. L, lens.

(A) Stage 41 retina lipofected with *pGFP*.

(B) Stage 41 retina lipofected with *p27Xic1*.

(C) GFP-positive Müller cell in a stage 41 control retina.

(D) GFP-positive Müller cell in a stage 41 retina lipofected with *p27Xic1*.

(E) Cells lipofected with *p27Xic1* in a stage 29/30 retina.

(F) Cells lipofected with *p27Xic1* in a stage 32 retina.

(G) Enlarged view of (D).

(H) Cells lipofected with pGFP in a stage 32 retina.

(I–K) R5 staining of stage 41 retina lipofected with *p27Xic1* plus pGFP. (I) *p27Xic1* lipofected cells. (J) R5 staining. (K) Overlapped view of (G) and (H).

(L) Immunostaining of *p27Xic1* lipofected retina with anti-calbindin antibody (red).

(M) Schematic drawing of *Xenopus* retina (modified from Dowling, 1987). ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; P, photoreceptor cells; H, horizontal cells, M, Müller cells; B, bipolar cells; A, amacrine cells; I, interplexiform cells; G, ganglion cells.

the amount of p27Xic1 RNA detected at different positions in the CMZ (Figure 1J). In agreement with the developmental analysis above, we find a gradual increase in p27Xic1 mRNA level from the periphery to the center of CMZ and then a sudden decrease near the end of CMZ (Figure 1J). Double in situ hybridizations show that *X-Notch-1* is expressed at about the same peripheral eccentricity as *p27Xic1*, indicating that it is expressed at about the same time (data not shown). However, *p27Xic1* is expressed more centrally (later) than *cyclin D1* (Figures 1K–1M) and more peripherally (earlier) than the proneural gene *Xath5* (Figures 1N–1P). To look at the relationship between cell division and *p27Xic1* expression, we injected stage 41 embryos with bromodeoxyuridine (BrdU) before fixation and then double-labeled them for BrdU and *p27Xic1* expression. p27Xic1 is clearly present in dividing cells in the CMZ, but high levels of expression also extend centrally into the postmitotic retina (Figures 1Q–1S). These expression patterns are summarized in Figure 1T.

p27Xic1 Overexpression Induces Müller Glia and Decreases Bipolar Neurons

To test whether p27Xic1 is involved in determination, we overexpressed p27Xic1 in developing retina by colipofection of a p27Xic1-expressing plasmid with a GFP-expressing plasmid. Misexpressing cells were identified by GFP fluorescence at stage 41, and cell types were initially classified on the basis of morphology and the position of nucleus in the laminar structure (Figure 2M). Using a plasmid driving a Myc-tagged p27Xic1 protein, we detected Myc immunoreactivity in 91.2% ± 1.0% of GFP-positive cells, indicating a high percentage of coexpression. Misexpression of p27Xic1 caused a dramatic increase in cells with Müller glia morphology. These cells of the inner nuclear layer have long bipolar processes with highly branched endfeet at the vitreal and ventricular surface of the retina (Figures 2B–2D). Forty percent of *p27Xic1* transfected cells became Müller glia, a 6-fold increase compared to retinas transfected with *GFP* alone (Figure 3A). There was a clear dose dependency of this effect, with over 60% of the cells expressing the highest level of Myc staining becoming Müller glia (Figure 3B). The Müller cell ratio was matched by a concomitant decrease in the ratio of bipolar cells (Figure 3A). This Müller inducing activity of p27Xic1 was totally unexpected, since we had assumed that a molecule causing early cell cycle arrest would bias retinoblasts toward the cell types that normally differentiate first, not those that differentiate last (i.e., ganglion cells not Müller cells).

To confirm the identity of these Müller cells, we stained transfected retinas with the Müller-specific antibody, R5 (Drager et al., 1984). p27Xic1-expressing cells with morphology of Müller cells were always double-labeled with R5 antibody (Figures 2I–2K). We also stained lipofected retinas with antibodies to other retinal

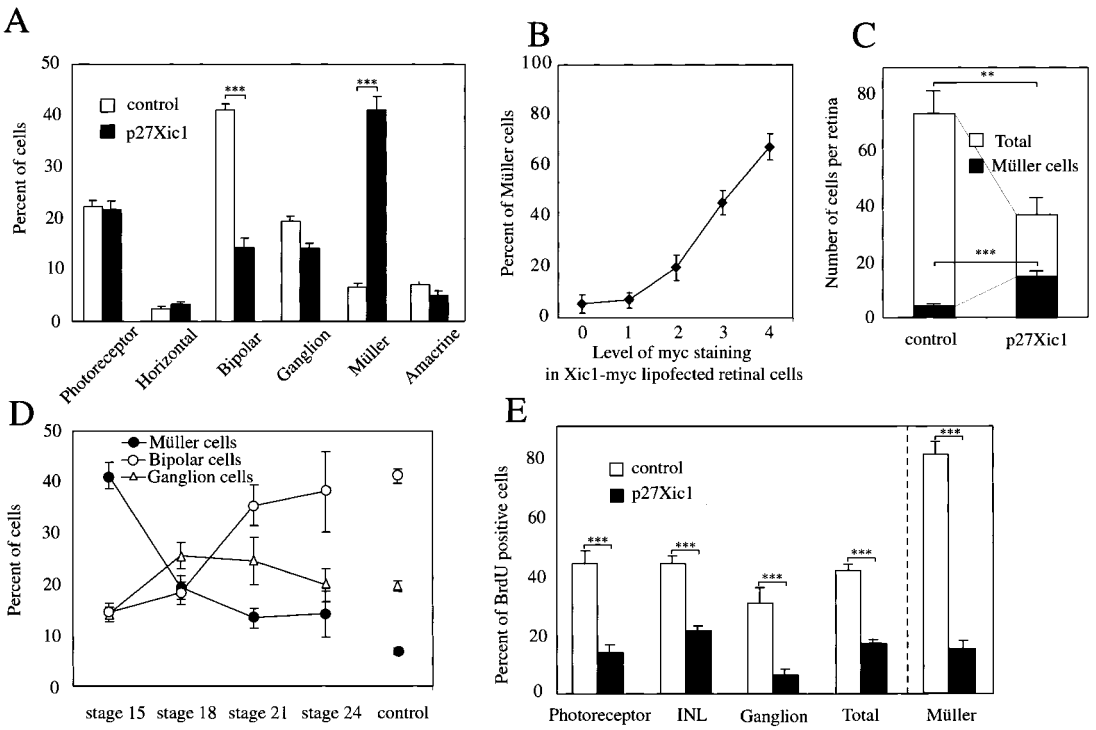

Figure 3. Overexpression of p27^{Xic1} Increases the Ratio of Müller Cells by Changing Cell Fate

(A) Percentage of retinal cell types labeled by misexpression of *p27^{Xic1}* plus pGFP or pGFP alone. The expression constructs were lipofected at stage 15, and the cell type was analyzed using sections of the stage 41 embryos. Inner plexiform cells are included in the category of amacrine cells. n = 675 cells (*p27^{Xic1}* plus pGFP) and n = 1764 (control). Error represents SEM; single asterisk, $p < 0.05$, double asterisks, $p < 0.01$, triple asterisks, $p < 0.001$ by Student's t test in all figures.

(B) Relationship between the expressed amount of p27^{Xic1} (Xic1-N-Myc) and ratio of Müller cells. After Myc staining of retina lipofected with *Xic1-N-Myc* and *GFP*, the relationship between an intensity of Myc staining and the ratio of Müller cells in GFP-positive cells was analyzed. Intensity of Myc staining was classified into five categories: 0, no Myc staining; 1, very weak; 2, weak; 3, medium; 4, strong.

(C) Total number of lipofected cells in retina and total number of Müller cells among the lipofected cells per retina.

(D) Müller cell induction depends on the lipofected stages. *p27^{Xic1}* was lipofected at the indicated stages, and the final differentiated cell types were analyzed at stage 41. A ratio in the retina lipofected with *pGFP* at stage 15 was used as the control because there is no stage dependency in the pGFP control.

(E) p27^{Xic1} causes early cell cycle arrest in the retina. Embryos were lipofected with *p27^{Xic1}* at stage 15. From stage 32, BrdU was injected into embryos, and at stage 41 the ratios of BrdU-positive cells in all lipofected cells were examined. INL, inner nuclear layer.

markers, including anti-calbindin (labeling cones, Figure 2L) (Chang and Harris, 1998), anti-rhodopsin (labeling rods), anti-glycine (labeling a subtype of amacrine cells), and anti-serotonin (labeling another subtype of amacrine cells). Although some lipofected cells expressed these markers, none of these had Müller cell morphology.

p27^{Xic1} overexpression primarily affects bipolar and Müller cells, two of the last cell types to differentiate in the retina, suggesting that the timing of p27^{Xic1} expression may have an effect on the resulting phenotype. To test this idea, we lipofected *p27^{Xic1}* at different stages and analyzed the effect on retinal differentiation (Figure 3D). The ratio of transfected cells that were Müller cells was decreased when the *p27^{Xic1}* was lipofected at a later stage (stage 21–24). The compensatory drop in bipolar cell was also diminished. In contrast, when *p27^{Xic1}* was lipofected earlier (stage 15), the ratio of Müller cells was increased, again mostly at the expense of bipolar cells.

p27^{Xic1} Causes Early Cell Cycle Arrest

One possible explanation for the increase in Müller cells among transfected cells is that p27^{Xic1} blocks proliferation in neural but not glial cell progenitors. Therefore, we compared the total number of transfected Müller cells after the colipofection with *p27^{Xic1}* and *GFP* with the total number of transfected Müller cells in retinas transfected with *GFP* alone (Figure 3C). The total number of GFP-positive Müller cells in the retinas colipofected with *p27^{Xic1}* and *GFP* (14.7 ± 1.89 cells/retina, n = 129 retinas) was about three times greater than the number of GFP-positive Müller cells lipofected with *GFP* alone (4.7 ± 0.51 cells/retina, n = 79). This increase in absolute number of Müller cells occurs, even though the total number of GFP-positive cells in the retina lipofected with *p27^{Xic1}* was decreased to half the number of the control. This indicates that p27^{Xic1} overexpression increases the absolute number of Müller cells while at the same time limiting retinal cell proliferation.

p27^{Xic1} overexpression might allow Müller cell precursors to divide but limit the proliferation of other cell types. To test the effect of p27^{Xic1} on retinal cell proliferation, embryos were colipofected with *p27^{Xic1}* and *GFP* at stage 15 and then injected with BrdU every 6 hr from stage 32 until stage 41. The ratio of BrdU incorporation in p27^{Xic1}-expressing cells was compared with BrdU incorporation in cells lipofected with *GFP* alone (Figure 3E). p27^{Xic1}-transfected cells showed dramatically

decreased ratios of BrdU incorporation. This decrease was most dramatic in Müller cells, as might be expected for the last born cell type (Figure 3E). These data indicate that p27Xic1 stops the cell cycle prematurely in all retinal cells.

p27Xic1 Does Not Cause Differential Apoptosis

It is known that Cdk inhibitors or dominant-negative Cdks suppress apoptosis and promote cell survival (Meikrantz and Schlegel, 1996; Park et al., 1997), raising the possibility that the effect of p27Xic1 on Müller cells is related to differential death of neurons over glia. Therefore, we examined the effect of p27Xic1 on apoptosis using a TUNEL assay (Gavrieli et al., 1992). Cell death was examined at stages 29, 31, 33/34, and 35/36. There was no difference in apoptosis between *p27Xic1*-transfected cells and the cells transfected with *GFP* alone at any stage examined, although the ratios slightly increase as embryos develop in all cases. About 3% of both *GFP* and *p27Xic1* transfected cells were TUNEL positive at stage 33/34.

p27Xic1 Converts Precursors from a Neural to a Glial Fate

Does p27Xic1 trigger Müller cell differentiation directly or indirectly through loss of competence to form neurons? Perhaps retinoblasts overexpressing p27Xic1 are unable to form neurons and forced to wait until glial determination begins before they differentiate. This possibility can be tested by asking when the p27Xic1-induced Müller cells differentiate. p27Xic1 lipofected cells were therefore examined at stages 29 and 32 when most cells in the retina are still undifferentiated (Figure 2H) (Holt et al., 1988; Dorsky et al., 1997; McFarlane et al., 1998). As early as stage 29, many cells lipofected with *p27Xic1* already have a single long process, with branched endfeet on the vitreal side and many branches on the pigmented epithelial side of the retina, characteristic of mature Müller cells and clearly different from simple tapered, neuroepithelial cells (Figure 2E). By stage 32, the branches become even more elaborate (Figures 2F and 2G). However, despite the Müller-like morphology, these cells did not stain with R5 antibody until stage 37/38 (data not shown).

Cycle Arrest Is Not Sufficient to Induce Müller Glia

Blocking the cell cycle by treatment of *Xenopus* embryos with hydroxyurea and aphidicolin (HUA) from stage 15 and 20 leads to the formation of eyes that have few but abnormally large cells. Interestingly, antibodies specific for retinal cell types indicate that most cell types are present in these cellularly depleted retinas (Harris and Hartenstein, 1991). To test whether these S phase inhibitors increased Müller glia, *GFP* was lipofected into the eye primordia at stage 15; HUA treatment was begun at stage 22; and the retinas were examined at stage 41. The ratio of R5-positive Müller cells that were GFP positive after HUA (8.84%, $n = 181$) is similar to that of nontreated control (7.37%, $n = 244$). Thus, cell cycle arrest at S phase with HUA is not sufficient for Müller cell induction.

The question then arises as to whether blocking cell division at a different point (e.g., the G1/S phase or M phase boundary) is able to induce Müller glia. We therefore lipofected retinas with dominant-negative forms of *Cdk2* and *cdc2*, which also block the cyclin kinase activity (Nebreda et al., 1995). These constructs did not induce Müller cells (data not shown).

The Cdk/Cyclin-Binding Region of p27Xic1 Is Involved in Müller Cell Induction, but Kinase Inhibitory Activity Is Not

p27Xic1 has three characteristic domains (Figure 4A). The N-terminal half contains a Cdk/cyclin-binding region, which is highly conserved in the mammalian Cdk inhibitors p21Cip1, p27Kip1, and p57Kip2 and is required for the members of Cip/Kip family to inhibit cyclin-dependent kinase activity. The C-terminal half of p27Xic1 contains a PCNA-binding region that is also found in p21Cip1 (Waga et al., 1994; Chen et al., 1995) and a potential cdc2 phosphorylation site, the QT domain that is also found in p27Kip1 (Polyak et al., 1994; Toyoshima and Hunter, 1994). In addition to its ability to block Cdk activity, interaction of p27Xic1 with PCNA can also inhibit DNA replication (Su et al., 1995). To find which region of p27Xic1 is responsible for Müller cell determination, two expression constructs were made: Xic1-N, which expresses N-terminal residues (1–96); and Xic1-C, which expresses the C-terminal residues (97–210) (Figure 4A). To confirm the biochemical function of these constructs, we examined the effect of each on cyclin-dependent kinase activity and on early embryonic cell division. As reported previously (Su et al., 1995), Xic1-N as well as full-length p27Xic1 inhibits Cdk kinase activity, as measured by the ability of the immunoprecipitated protein to phosphorylate histone H1, but Xic1-C does not (data not shown). When RNA was injected into a single blastomere of two-cell stage embryos, Xic1-N inhibited cell division on the injected side shortly after injection, whereas the blastomeres of the uninjected side still divided normally (Figure 4D). In contrast, Xic1-C, which acts by inhibiting PCNA-dependent replication, did not stop cell division before stage 8.5, corresponding to the mid-blastula transition (MBT). After the MBT, however, the cells on the Xic1-C-injected side were larger, indicating inhibition of cell cycle. By late stage 9, the cells on this side were about twice the size of those on the uninjected side (Figures 4F–4H). Thus, both Xic1-N and Xic1-C can stop the cell cycle, but by different mechanisms, at different embryonic stages, and at different points in the cell cycle. Retinal lipofection of Xic1-N resulted in almost the same amount of Müller cell inducing activity as full-length p27Xic1, whereas Xic1-C lipofection had no effect on the Müller cell ratio (Figure 4I), even though all p27Xic1 constructs arrested the cell cycle in the retina with equal potency (Figure 4J).

In an attempt to rescue Müller cell induction by p27Xic1, we lipofected different types of *Cdk*s (*Cdk2*, *Cdk4*, and *Cdk5*) with *p27Xic1* into the retina and analyzed induced Müller cell number. All Cdks decreased the ratio of Müller cells induced by p27Xic1 (data not shown), but the effect of Cdk2 was the strongest (Figure 4K). Cdk2 and cyclin A2 form a complex and bind to different parts of p27Kip1 (Russo et al., 1996). The inhibitory effect of colipofecting the combination of Cdk2 and cyclin A2 on the p27Xic1-induced phenotype was stronger than that of either Cdk2 or cyclin A2 alone (Figure 4K). How these

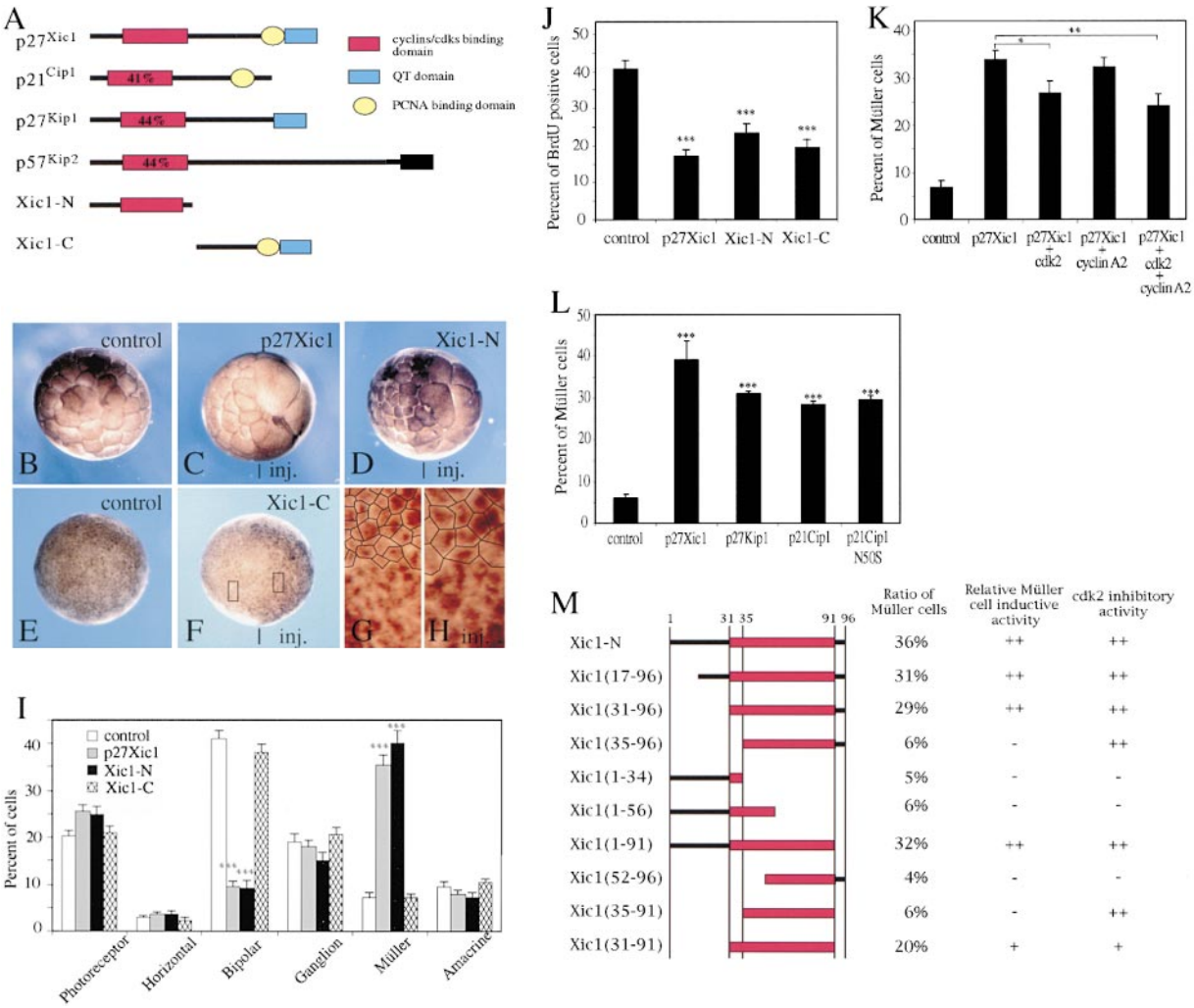

Figure 4. p27^{Xic1} Induces Müller Cells through the N-Terminal Domain

(A) Schematic structures of p27^{Xic1}, p21^{Cip1}, p27^{Kip1}, p57^{Kip2}, Xic1-N, and Xic1-C. Homology of cyclin/Cdk-binding domain of p27^{Xic1} with those of mammalian homologs is indicated in the box.

(B–H) mRNA-injected early embryos. mRNA (2 ng) of p27^{Xic1} (C), Xic1-N (D), Xic1-C (F, G, and H), or mock (B and E) was injected in a blastomere of two-cell stage embryos. The effect was observed at 64-cell stage (B–D) or at stage 9 (E–H). (G and H) Enlarged views of the indicated region in (F). Outlines of cells were marked. inj., injected side.

(I) Percentage of retinal cell types labeled by misexpression of p27^{Xic1}, Xic1-N, or Xic1-C. The expression construct of p27^{Xic1}, Xic1-N, Xic1-C, or GFP was lipofected at stage 15, and then the ratios of retinal cell types were determined at stage 41.

(J) Xic1-N and Xic1-C cause early cell cycle arrest in the retina. Embryos were lipofected with *p27^{Xic1}*, *Xic1-N*, *Xic1-C*, or *pGFP* at stage 15. From stage 32, BrdU was injected into embryos, and at stage 41 the ratios of BrdU-positive cells in retina were examined.

(K) Cdk2 and cyclin A2 partially inhibit the inductive effect of p27^{Xic1}. Expression construct of Cdk2 or cyclin A2 was colipofected with *p27^{Xic1}* at stage 15, and the cell types were analyzed at stage 41.

(L) p27^{Kip1}, p21^{Cip1}, and p21^{Cip1} N50S also induce Müller cells. Expression construct of p27^{Kip1}, p21^{Cip1}, or p21^{Cip1} N50S was lipofected at stage 15 and then the cell types were analyzed at stage 41.

(M) Müller cell inductive activities and Cdk2 inhibitory activities of p27^{Xic1} deletion constructs. The structures of the constructs are indicated at the left side. The constructs were lipofected at stage 15. The ratio of Müller cells was analyzed at stage 41. Cdk2 kinase inhibitory activity was measured as described in Experimental Procedures. Relative Müller cell inductive activity and Cdk inhibitory activity were classified into three categories: −, no activity; +, weak; ++, strong.

cyclins abrogate p27^{Xic1} activity is not clear. They may bind and deplete active p27^{Xic1} or block the Müller-inducing site of the N-terminal domain.

The mammalian homologs p21^{Cip1} and p27^{Kip1} share the Cdk/cyclin-binding domain with p27^{Xic1}, and both constructs increased the ratio of Müller cells (Figure 4L). A point mutant, N50S, of p21^{Cip1} has minimal kinase inhibitory activity, although it still promotes assembly of cyclin D and Cdk4 (Welcker et al., 1998). Surprisingly, N50S induced Müller cells (Figure 4L), although it could not stop cell division before MBT when the mRNA was injected in one blastomere of two-cell stage embryos (data not shown) and could not inhibit Cdk2 kinase activity as assayed by H1 kinase assay. Wild-type p21^{Cip1} inhibits 96.1% of Cdk2 kinase activity, while N50S inhibits only 15.6% of this activity. These results suggest

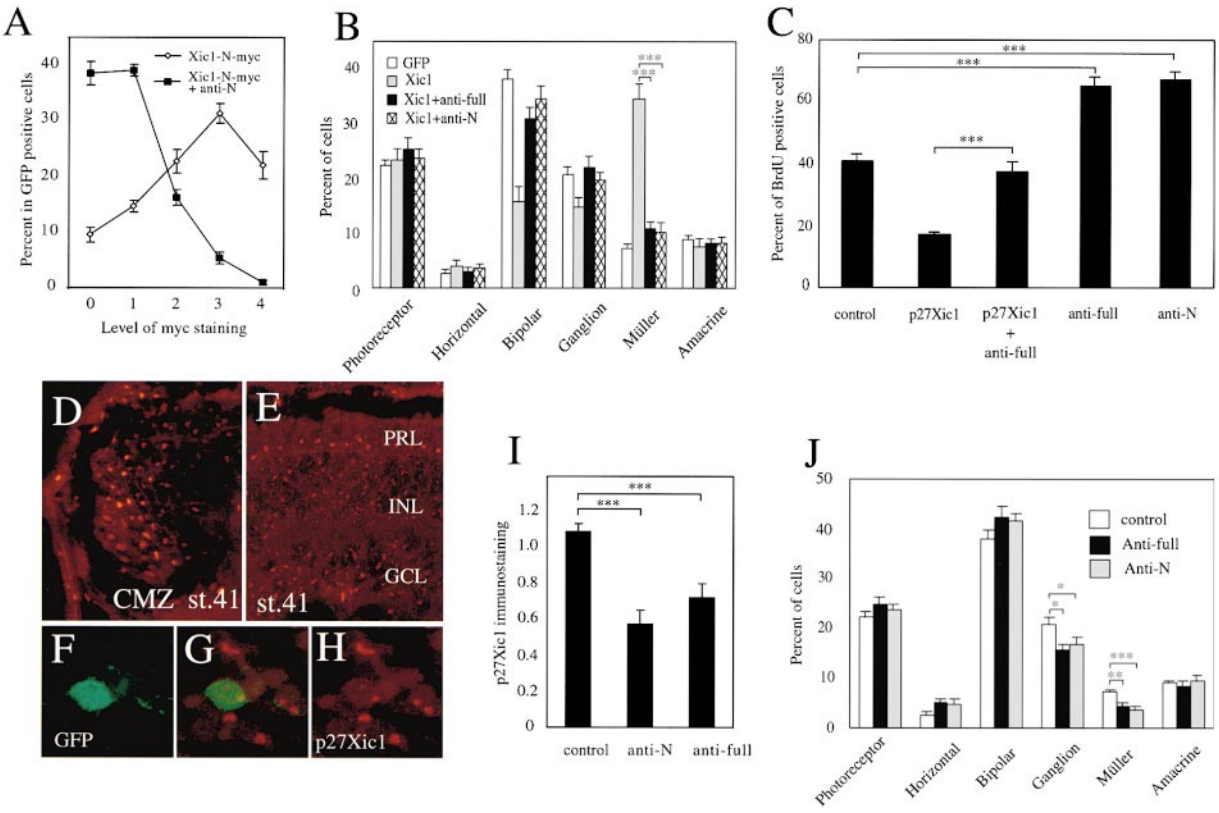

Figure 5. Expression of p27Xic1 Antisense DNA Results in a Decrease of Müller Cells

(A) Antisense construct lipofection decreases an expression of p27Xic1 protein from the lipofected p27Xic1-Myc plasmid. Retinas were lipofected with *Xic-N-Myc* (sense) plus *anti-N* (antisense) and *pGFP* or with *Xic-N-Myc* plus *pGFP* at stage 15. After the Myc staining of sections from stage 41 embryos, intensities of Myc staining in GFP-positive cells were compared. Intensity of Myc staining was classified into five categories: 0, no Myc staining; 1, very weak; 2, weak; 3, medium; 4, strong.

(B) Antisense *p27Xic1* DNA can inhibit the inductive effect of the sense construct. An antisense *p27Xic1* construct of anti-full or anti-N was colipofected with *p27Xic1* in a 3:1 ratio, and then the cell types were analyzed at stage 41.

(C) Function of antisense constructs on cell cycle arrest in the retina. The indicated constructs were lipofected with *pGFP* at stage 15. From stage 32, BrdU was injected into embryos, and at stage 41 the ratios of BrdU-positive cells that were also GFP positive were examined.

(D and E) Immunostaining with p27Xic1 antibody. (D) CMZ of stage 41 retina. (E) A central region of a stage 41 retina. PRL, photoreceptor layer; INL, inner nuclear layer; GCL, ganglion cell layer.

(F–I) The antisense construct decreases endogenous p27Xic1 expression. The antisense construct with *pGFP-Myc* was lipofected at stage 15. Sections of stage 32 retina were immunostained with p27Xic1 antibody and Myc antibody (9E10). (F–H) Retina colipofected with anti-N and pGFP. (F) GFP (Myc staining). (G) Overlapped views. (H) p27Xic1 antibody staining. (I) The intensities of p27Xic1 antibody staining were measured using Openlab 2.0.8 (Improvision) by comparing the relative values of p27Xic1 intensity in GFP-positive cells to surrounding untransfected cells (control, lipofected cells, n = 19, neighbors, n = 59; anti-N, lipofected cells, n = 16, neighbors, n = 50; anti-full, lipofected cells, n = 10, neighbors, n = 32).

(J) Antisense constructs of *p27Xic1* inhibit natural Müller cell differentiation. An antisense *p27Xic1* construct of anti-full or anti-N was lipofected at stage 15, and then the cell types were analyzed at stage 41.

that p27Xic1 determines the Müller cell fate in a manner independent of the kinase inhibitory activity and cell cycle activity of the N-terminal domain.

To map the Müller-inducing portion of Xic1-N, several deletion constructs were assayed for Cdk2 inhibitory activity and Müller cell inducing activity (Figure 4M). Animo acids 31–35 were found to be essential for Müller cell inducing activity, while amino acids 35–91 were critical for both kinase inhibitory activity and Müller cell inducing activity. Thus, constructs Xic1(35–96) and Xic1(35–91) had kinase inhibitory activity but no Müller cell inducing activity, opposite to N50S, which had Müller cell inducing activity but no kinase inhibitory activity. This analysis functionally separates these activities to different regions of the N-terminal domain.

Endogenous p27Xic1 Works as a Müller Cell Determinant in Normal Development

An important question is whether the endogenous p27Xic1 in the retina also works as a Müller determinant. Full-length p27Kip1 antisense cDNA can reduce the amount of the p27Kip1 protein by up to 90% in cell culture (Rivard et al., 1996). Consequently, retinal cells were cotransfected with Myc-tagged p27Xic1 and an antisense construct at a ratio of 1:3. These cells showed a dramatic decrease in Myc expression (Figure 5A). When either full-length or N-terminal antisense was lipofected with the *p27Xic1* sense DNA, the induction of Müller cells was inhibited by about 85% (Figure 5B). Both antisense constructs also inhibited cell cycle arrest, as evidenced by increased BrdU incorporation in *p27Xic1* cotransfected cells (Figure 5C).

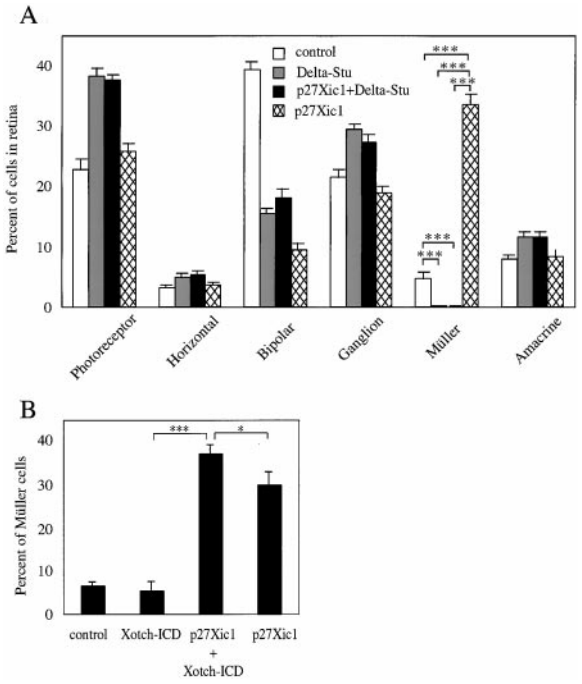

Figure 6. p27^{Xic1} Works within the Context of an Active Neurogenic Pathway

(A) Dominant-negative Delta can overcome the effect of p27^{Xic1} on Müller cell determination. The expression constructs *p27^{Xic1}*, *Delta-stu*, or both were lipofected at stage 15. The cell type was analyzed using sections of stage 41 embryos.

(B) Constitutive active X-Notch-1, Xotch-ICD enhances the inductive activity of p27^{Xic1}. The expression constructs *p27^{Xic1}*, *Xotch-ICD*, or both were lipofected at stage 15. Cell type was analyzed using sections of stage 41 embryos.

We then examined the effect of the antisense constructs on endogenous Müller cell differentiation. To demonstrate that the antisense lowered native protein level, we used an affinity-purified anti-Xic1 antibody, which labels the nuclei of p27^{Xic1}-expressing cells. In the stage 41 retina, as expected, the CMZ is more heavily labeled than the central retina (Figures 5D and 5E). Cells transfected with antisense and GFP constructs showed a marked reduction in p27^{Xic1} protein expression compared to cells transfected with GFP alone (Figures 5F–5I). After lipofection with the antisense constructs at stage 15, embryos were allowed to develop until stage 41, and the fraction of Müller glia cells compared to GFP controls was determined. Only 4% of antisense lipofected cells became Müller cells, about half the control fraction (Figure 5J). BrdU analysis showed that antisense transfected cells also tended to stay in the cell cycle longer than controls (Figure 5C). Antisense transfection had no affect on cell death at any stage examined (data not shown).

p27^{Xic1}-Induced Müller Cell Differentiation Operates within the Context of the Notch Pathway

It is clearly interesting to investigate whether p27^{Xic1} can interact with other proteins primarily involved in determination events, such as Notch and Delta (Dorsky et al., 1995, 1997; Henrique et al., 1997). Delta-stu, a dominant-negative form of X-Delta-1, blocks the Notch/Delta pathway. Strikingly, *Delta-stu* colipofected with *p27^{Xic1}* at stage 15 completely inhibited the Müller inducing activity of p27^{Xic1} (Figure 6A). To activate the Notch/Delta pathway, we used the constitutively active intracellular domain of X-Notch-1 (Notch-ICD) (Dorsky et al., 1995). When it is colipofected with *p27^{Xic1}*, Müller glia are induced at even higher levels than by *p27^{Xic1}* alone (Figure 6B). These results suggest that p27^{Xic1} works as a Müller cell determinant in the context of an active Notch/Delta pathway. This idea fits well with the fact that Müller glia are the last cells in the *Xenopus* retina to express Notch-1 during embryonic development.

Discussion

p27^{Xic1} as an Intrinsic Determinant of Retinal Cell Fate

All retinal neurons and Müller glial cells are formed from the multipotential precursor cells in a conserved order. This sequential appearance is regulated by the interplay of extrinsic and intrinsic factors, many of which have been identified (reviewed in Harris, 1997). During development in *Xenopus*, retinal precursors lose their multipotency (Harris, 1997). Belliveau and Cepko (1999) showed that late rat retinal progenitors are restricted to producing late cell types even when transplanted into early retinas. Similarly, early precursors do not normally produce late cell types until they are scheduled to do so, even when they are mixed with late cells (Watanabe and Raff, 1990; Belliveau and Cepko, 1999). This indicates that a temporal maturation of retinal precursor cells, possibly involving the build up of intrinsic factors, is necessary to confer specific fates. In this paper, we demonstrate that expression of p27^{Xic1} increases during retinal cell development, that overexpression of p27^{Xic1} in the precursor cells can induce Müller cells, and that reducing p27^{Xic1} function causes a decrease of Müller cells. These results clearly support the role of p27^{Xic1} as a positively acting intrinsic factor of the type predicted.

Direct and Indirect Pathways of Cellular Induction in the Retina

An increased population of Müller glia cells can occur as a consequence of inhibition of pathways affecting neural determination. For example, FGF2 expressed in the retina induces differentiation into retinal ganglion cells (Zhao and Barnstable, 1996), while inhibition of the FGF signal by overexpression of a dominant-negative FGF receptor increases the number of Müller cells (McFarlane et al., 1998). Likewise, a loss of NeuroD function, which is involved in the differentiation and survival of retinal neurons, also causes an increase in Müller cells (Morrow et al., 1999). TGFα is thought to increase the number of Müller cells because it promotes mitotic activity, which, in turn, delays entry into a competent postmitotic state until rod inducing activity has become weak (Lillien and Wancio, 1998). In all of these cases, it seems likely that the increase in Müller glia is secondary to the action of these factors on other cells types. However, the fact that overexpression of p27^{Xic1} can induce the precocious development of Müller cells suggests a more direct action on glial cell determination. If p27^{Xic1} directly induces glial cells, the decrease in bipolar cells may be a secondary consequence, although a direct effect of p27^{Xic1} on bipolar neuron differentiation cannot be ruled out by the experiments presented here. The

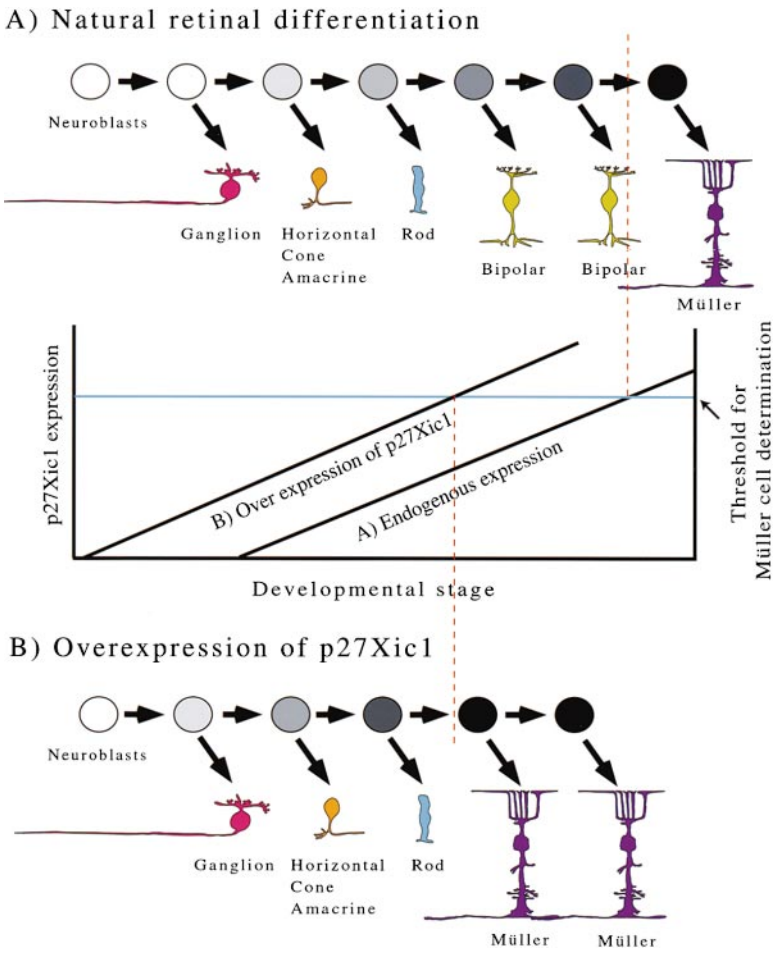

Figure 7. Intrinsic Timer Model

A gradual increase of p27Xic1 can define the timing of the Müller cell fate decision in the sequence of retinal cell differentiation. The accumulation of p27Xic1 in the normal retina is indicated in the central graph and by the increasing level of shading in the line of precursors in (A). If cells remain in the cell cycle until p27Xic1 expression reaches a threshold level, this increases the probability both of exiting the cell cycle and adopting a Müller cell fate (dashed line up). (B) Overexpression of p27Xic1 by lipofection causes early accumulation of the protein in precursors, resulting in early cell cycle arrest and differentiation of Müller cells at the expense of bipolar cells (dashed line down).

bipolar fate seems particularly labile in a number of experimental systems and is often found compensating for gains and losses in photoreceptor number (Dorsky et al., 1997; Ezzeddine et al., 1997; Kanekar et al., 1997).

Competence to respond to extrinsic determination factors changes during the cell cycle (McConnell and Kaznowski, 1991; Ericson et al., 1996; Bohner et al., 1997; Belliveau and Cepko, 1999). The phase of the cell cycle may be an important factor in regulating the competence of precursors to respond to extrinsic determinants. Although it is not clear when p27Xic1 determines cell fate during the cell cycle, our results suggest some interesting possibilities. Since the inductive activity of p27Xic1 on Müller cell differentiation is modulated by cyclins and Cdks, these components might also regulate the competence to respond to other differentiation factors via the function of p27Xic1. It will be interesting in this regard to learn more about the possible function of amino acids 31–35 of p27Xic1 with respect to cyclin and Cdk binding.

Mechanism of Determination of Müller Cell Fate by p27Xic1

How does p27Xic1 induce the Müller cells, the last cell type in a normal differentiation scheme? We propose an intrinsic timer model (Figure 7A). The cell fate decision to become Müller cells is influenced by endogenous expression of p27Xic1, which gradually increases during retinal cell development, competing with other differentiation factors, so that the last cells to be born generally have the most p27Xic1. If the Müller cell determining activity of p27Xic1, expressed at high levels, overcomes the activity of other determinants, these cells will become committed to a Müller fate. Such a situation is reached early in *p27Xic1* lipofected retinas, resulting in an increase in the ratio of Müller glia (Figure 7B). A similar model has been proposed to explain the function of p27Kip1 on oligodendrocyte differentiation (Durand et al., 1998). It is particularly revealing that the influence of p27Xic1 on Müller cells, the last cell type in the retina, is gated by the activity of the Delta/Notch pathway, which inhibits the determination of early cells types. The Notch pathway, the cell cycle, and cell determination are thus interconnected by the functions of p27Xic1.

Experimental Procedures

Xenopus Embryos

Embryos obtained by in vitro fertilization were dejellied in 2% cysteine (pH 8.0) and allowed to develop in 0.1× MBS using standard methods. Embryos were staged according to Nieuwkoop and Faber (1994).

Constructs

Xenopus Cdk4 and *cyclin D1* were kindly provided by Dr. Tim Hunt (ICRF, UK). *Xenopus p27Xic1* (Su et al., 1995), *Cdk2* (Paris et al., 1991), *Cdk4*, *Cdk5* (Philpott et al., 1997), *cdc2* (Pickham et al., 1992), *cyclin*

A2 (Howe et al., 1995), *cyclin D1*, human *p21^{CIP1}* (Harper et al., 1993), human *p21^{CIP1}* N50S (Welcker et al., 1998), and mouse *p27^{Kip1}* (Toyoshima and Hunter, 1994) were cloned into pCS2$^+$ (Turner and Weintraub, 1994) for expression in *Xenopus*. All deletion constructs, including p27^{Xic1} N-terminal half (Xic1-N; residues 1–96) and C-terminal half (Xic1-C; residues 97–210), and antisense constructs for full-length p27^{Xic1}, anti-full (full coding sequence), and 5′ region of coding sequence of p27^{Xic1}, anti-N (nucleotides 0–393) were also made using pCS2$^+$.

In Situ Hybridization of Whole Embryos and Retinal Sections

Digoxigenin (DIG)-labeled antisense RNA probes of *p27^{Xic1}*, *cyclin D1*, *cyclin A2*, *Cdk4*, *Cdk2*, and *cdc2* and the fluorescein-labeled antisense RNA probes of *p27^{Xic1}*, *X-Notch-1*, and *Xath5* were produced from the T7 or T3 promoter of pCS2$^+$. Whole-mount in situ hybridizations on albino *Xenopus* embryos were performed as described (Harland, 1991). Where appropriate, in situ hybridizations were performed on 10 mm paraffin section of *Xenopus* embryonic eyes at stage 42 as described (Perron et al., 1998).

Immunohistochemistry

Immunostaining was carried out using 10 μm cryostat sections. Primary antibodies used in this study were as follows: monoclonal mouse anti-Myc (9E10) at a dilution of 1:1000 to stain Myc-tagged proteins (Sigma), anti-calbindin (Sigma) at 1:200 to identify cone photoreceptors, anti-rhodopsin at 1:1 to identify rod photoreceptors (Dr. Paul Hargrave), anti-glycine at 1:1 to identify a subtype of amacrine cells, anti-serotonin at 1:1 to identify a subtype of amacrine cells (INCSTAR), and anti-R5 at 1:1 to identify Müller cells (Drager et al., 1984). Primary antibodies were detected using Cy3-conjugated donkey anti-mouse IgG or IgM or TRITC-conjugated goat anti-rabbit IgG (Jackson Laboratories) at a dilution of 1:500. Polyclonal antibodies to p27^{Xic1} were raised in rabbits against the peptide CPLEQTPRKKIR (Shou and Dunphy, 1996) and affinity purified using the Sulpholink kit (Pierce). After treatment of sections with 0.1% SDS at 95°C for 5 min to unmask the p27^{Xic1} epitope, they were stained with the purified p27^{Xic1} antibody.

Lipofection

pCS2$^+$ constructs were lipofected into eye primordia of embryos at stage 15, 18, 21, or 23 essentially according to the methods as described (Holt et al., 1990). The target regions of lipofection at stage 15 and stage 18 were determined from the fate map by Eagleson and Harris (1990) and from the expression region of *Pax6* and *XOptx2*, which are expressed in the eye primordium (Zuber et al., 1999). At stage 21 and 23, the eye primordia are easily recognized. *GFP* was cloned into pCS2$^+$ (pGFP) and colipofected to mark transfected cells. In double and triple lipofection, to normalize the amount of DNA introduced, pCS2$^+$ was colipofected.

mRNA Injection into an Early Blastomere

Capped RNAs were synthesized in vitro from *p27^{Xic1}*, Xic1-N, Xic1-C, and other mutants of p27^{Xic1}, p21^{Cip1}, and p21^{Cip1} N50S template DNAs using the SP6 Message Machine kit (Ambion). Two nanograms of RNA was injected in a volume of 10 nl into one blastomere of two-cell stage embryos. One half of the embryo expresses the protein from the injected RNA, and the other half of the embryo serves as an uninjected control. Cleavage was analyzed 2 hr later after the injection and at stage 9. The H1 kinase assays were performed as described (Philpott et al., 1997).

BrdU Staining

p27^{Xic1} plasmid along with *pGFP* or *pGFP* alone was lipofected into eye primordia of embryos at stage 15. After aging until stage 32, embryos were injected with BrdU intraabdominally every 6 hr. The embryos were fixed with 4% paraformaldehyde at stage 41, and cryostat sections were made through the head. BrdU incorporation was detected as described (Perron et al., 1998). The ratio of BrdU-positive cells that were also GFP positive was determined in the three cell layers of retina. For Figures 1Q–1S, BrdU was injected at stage 41. After fixation of the embryos an hour later, BrdU staining and p27^{Xic1} in situ hybridization were performed as described above.

TUNEL Assay

p27^{Xic1} along with *pGFP*, *anti-N* with *pGFP*, or *pGFP* only was lipofected into eye primordia of embryos at stage 15. After aging until stage 29, 31, 33/34, or 37/38, embryos were fixed with 4% paraformaldehyde and cryostat sectioned. Apoptotic cells were detected using the Apoptosis Assay System (Promega) with the following modification: Red Nucleotide mix (50 mM FluoroRed [Amersham], 100 μM dATP, 10 mM Tris-HCl [pH 7.6], 1 mM EDTA) was substituted for the Nucleotide Mix in the kit.

Hydroxyurea and Aphidicolin Treatment

pGFP was lipofected into the eye primordia of embryos at stage 15. The lipofected embryos were treated with a mixture of 20 mM hydroxyurea and 150 mM aphidicolin in 0.1× MBS solution adjusted to pH 7.4 from stage 22 to stage 41, as estimated from the control embryos as described (Harris and Hartenstein, 1991). After fixing with 4% paraformaldehyde and cryostat sectioning, sections were stained with R5 antibody. The ratio of R5-positive cells in GFP-positive cells was compared with that of the untreated embryos.

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